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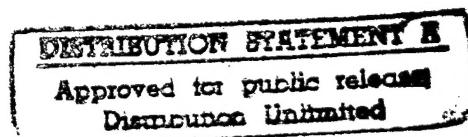
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Stereochemical Determination of Selegiline Metabolites in Post- mortem Biological Specimens

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16. Abstract The Federal Aviation Administration's Toxicology and Accident Research Laboratory determines the presence of drugs, volatiles, and primary combustion gases in biological samples from aircraft accident victims and also establishes any medical condition for which the drugs might have been taken. In this study, findings related to an aircraft accident are reported. Along with biological specimens from the pilot of this fatal accident, two types of tablets found at the accident scene were submitted for analysis. These tablets were identified as levodopa and selegiline, commonly prescribed for the treatment of Parkinson's disease. Selegiline, a stereospecific compound, is biotransformed into (-)-N-desmethylselegiline, (-)-methamphetamine, and (-)-amphetamine. During this process, the chiral center of the parent molecule is not affected. The latter two <i>levorotatory</i> metabolites cannot be easily distinguished by routine analysis from their <i>dextrorotatory</i> isomers, which are controlled substances. Therefore, it was prudent to differentiate these isomers to prove or disprove the controlled substance categorization. Initial immunoassay drug screenings revealed the presence of amphetamine class drugs (867 ng/ml) and amphetamine/methamphetamine (261 ng/ml) in urine and methamphetamine (46 ng/ml) in blood. The gas chromatography-mass spectrometry (GC/MS) results revealed the presence of methamphetamine in the concentrations of 76 ng/ml of blood and 685 ng/ml of urine. The level of amphetamine was 52 ng/ml in blood and 320 ng/ml in urine. To determine the stereospecificity of these amines, the isolated amines from the biosamples were derivatized by a stereospecific agent, (S)-(-)-N-(trifluoroacetyl)prolyl chloride, and characterized by a GC/MS method to be <i>levorotatory</i> . The 2.14 ratio of (-)-methamphetamine to (-)-amphetamine concentrations in the urine was consistent with a selegiline study in the recent literature. The stereospecific analysis, in conjunction with the history of the pilot being on Parkinson's medications, suggests that the source of these amines was selegiline. This conclusion substantiates the importance of the identification of enantiomers in evaluating and interpreting related analytical results for accident investigations.			
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STEREOCHEMICAL DETERMINATION OF SELEGILINE METABOLITES IN POSTMORTEM BIOLOGICAL SPECIMENS

INTRODUCTION

To facilitate aircraft accident investigations, biological samples collected from the aircraft accident victims are submitted to the Federal Aviation Administration's (FAA's) Toxicology and Accident Research Laboratory for toxicological evaluation. In coordination with the FAA's Office of Accident Investigation, these samples are submitted to the laboratory through the National Transportation Safety Board. This board is responsible for determining the cause of all United States civilian aircraft (air carrier and general aviation) crashes. The submitted biological samples are analyzed for prescription, nonprescription, and illicit drugs, along with volatiles and primary combustion gases.

In the present study, analytical findings related to a unique general aviation aircraft accident are reported. The four-seat aircraft (Mooney M20J) with one occupant impacted the ground near the runway and was destroyed; the 68 year-old male pilot received fatal injuries. At the time of the accident, the meteorological conditions were normal. At the crash site, two types of tablets were found. These tablets and the biological samples from the victim were submitted to the laboratory for analysis. The tablets were identified as levodopa and selegiline by their shape, color, and markings. This combination of drugs is routinely prescribed for the treatment of Parkinson's disease. The pilot had a history of this disease.

During the course of Parkinson's pathogenesis, the brain levels of the endogenous dopamine neurotransmitter decrease (Rall, 1990; Cooper et al., 1991). Both medications increase the levels of dopamine in the brain. Levodopa acts as the precursor of dopamine (Cooper et al., 1991), while selegiline is a monoamine oxidase inhibitor (USP DI®, 1991). The inhibition of the enzyme prevents the metabolism of endogenous dopamine, thereby increasing its levels in the brain.

Selegiline [*(R)*-(-)-*N*, α -dimethyl-*N*-(prop-2-ynyl)phenylethylamine] is rapidly absorbed from the gastrointestinal tract. The drug easily crosses the blood-brain barrier and is extensively biotransformed into (-)-*N*-desmethylselegiline, (-)-methamphetamine, and (-)-amphetamine (Figure 1). Because the chiral center of selegiline is not affected during its metabolism, the three main metabolites remain in the *levorotatory* isomeric [(-) isomeric] forms (Meeker and Reynolds, 1990). *Dextrorotatory* amphetamines—(+)-amphetamine and (+)-methamphetamine—have different pharmacological effects than their respective *levorotatory* isomers and are considered to be controlled substances. Therefore, it was essential to analytically differentiate the *levorotatory* isomers [(-) isomers] from the *dextrorotatory* isomers [(+) isomers].

Routine analyses were performed on the submitted biological samples for the spectrum of analytes, including amphetamine and methamphetamine. These amines were further stereospecifically analyzed and characterized to establish that these amines were *levorotatory*, originating from selegiline. No attempts were made to perform the analyses for selegiline and its desmethyl metabolite because selegiline is not present in appreciable amounts *in vivo* (Meeker and Reynolds, 1990), and the reference standard for the desmethyl product was not easily available. It was also not feasible to conduct analysis for levodopa to distinguish ingested levodopa from the endogenous levodopa.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade and solvents were of HPLC grade. These chemicals, immunoassay kits, standards, internal standards, and derivatizing

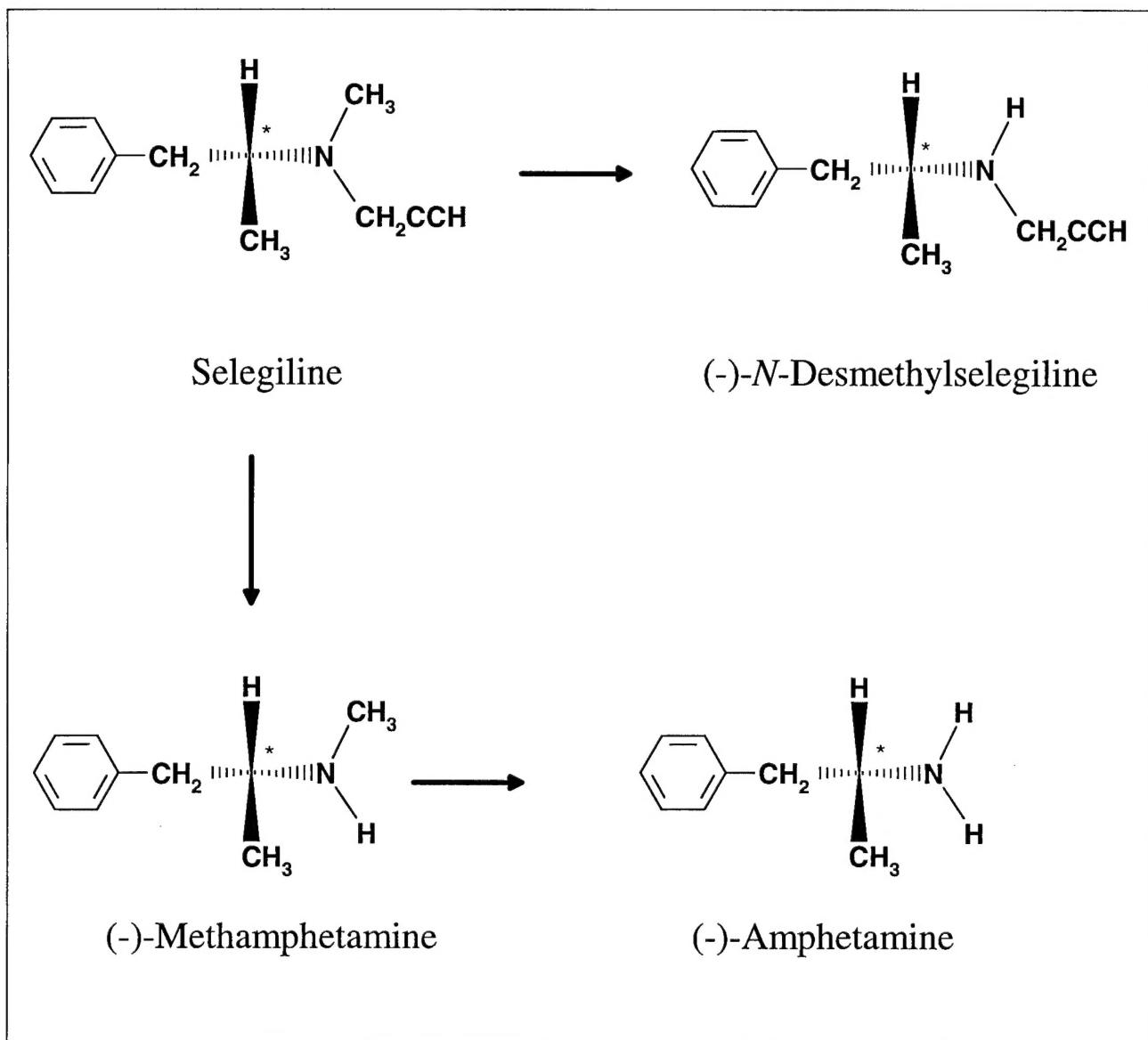


Figure 1. Metabolism of selegiline. The chiral centers (asymmetric carbons) are depicted by asterisks.

agents were obtained from commercial sources. The immunoassay kits for amphetamine/methamphetamine screens were purchased from Abbott Laboratories (Abbott Park, IL) and from Roche Diagnostic Systems (Nutley, NJ). Standards of (+)- and (-)-amphetamines and of (+)- and (-)-methamphetamines were obtained in methanolic solutions from Alltech-Applied Science Labs (State College, PA). The internal standards were supplied as racemic mixtures, (\pm)-amphetamine- d_8 and (\pm)-methamphetamine- d_8 , by Radian International LLC (Austin, TX). Pentafluoropropionic anhydride (PFPA) was purchased from Pierce (Rockford, IL). The chiral probe used in resolving these enantiomers was (*S*)-(-)-*N*-(trifluoroacetyl) prolyl chloride (TPC). Obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI), this probe was 97% enantiomerically pure and was supplied as 0.1 M solution in dichloromethane.

Screening

Blood and urine samples were screened and analyzed for common analytes using our laboratory's routine, standard, established procedures. These procedures entailed analyses for volatiles, carboxyhemoglobin, cyanide, and drugs using various chromatographic and spectroscopic techniques, as well as enzymatic and radioactive immunoassays. The urine sample was screened for amphetamine and methamphetamine by the Abbott's ADx® Amphetamine Class and ADx® Amphetamine/Methamphetamine II assays. Both assays are based on fluorescence polarization immunoassay technology. The blood sample was examined by the Roche's abuscreen® radioimmunoassay to detect methamphetamine. These assays provided semiquantitative levels of these amines in the biological samples. Although the Roche's radioimmunoassay is considered highly specific for methamphetamine, cross reactivities have been reported with amphetamine-like compounds, including their corresponding *levorotatory* and *dextrorotatory* isomers.

Quantitative Analysis of Amphetamine and Methamphetamine

Extraction

Based on the screening findings, the blood and urine samples were further tested for the confirmation and quantitation of amines. This testing was achieved by using our laboratory's standard procedure for the isolation of amines by solid phase extraction (SPE) technique. Four ml of blood and 4 ml of urine were separately spiked with 400 ng of each of the internal standards (\pm)-amphetamine- d_8 and (\pm)-methamphetamine- d_8 . To the spiked blood sample, 6 ml of acetonitrile was added to precipitate the proteins. Subsequently, the tube was capped, shaken for 5 minutes, and centrifuged at 120 \times g for 5 minutes. The liquid phase was transferred into a new tube and was evaporated to less than 1 ml using a stream of nitrogen; then, 2 ml of 0.1 M phosphate buffer (pH 6) was added to the tube. This acetonitrile-mediated precipitation was not necessary for the urine specimen, wherein 2 ml of the 0.1 M phosphate buffer was directly added.

The SPE columns were first conditioned with 2 ml of methanol, followed by 2 ml of the phosphate buffer. The previously buffered blood extract and urine sample were transferred onto the conditioned columns and allowed to pass through. The columns were rinsed with 1 ml of 1.0 M acetic acid and dried under vacuum (15 inches Hg) for 5 minutes. Following this, 6 ml of methanol was passed through the columns, and then they were allowed to dry for 2 minutes. The analytes were eluted from the columns into tubes using 4 ml of 2% ammonium hydroxide in ethyl acetate. Hydrochloric acid gas was bubbled through the collected eluates to minimize loss of amines.

Derivatization by PFPA

The obtained eluates were evaporated to dryness using a stream of nitrogen, and 50 μ l of ethyl acetate and 50 μ l of PFPA were added to the residues. The

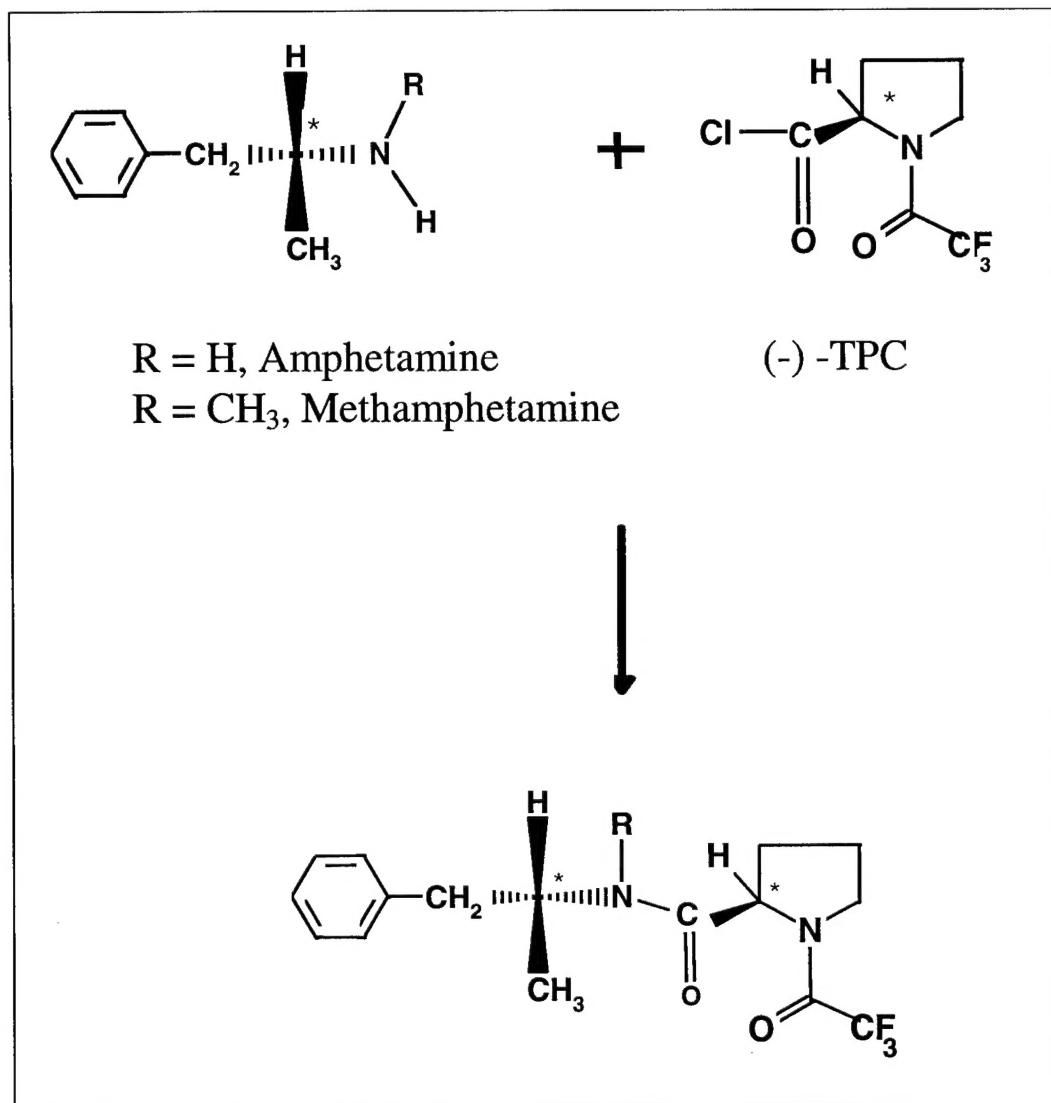


Figure 2. Derivatization of amphetamine and methamphetamine by (S)-(-)-N-(trifluoroacetyl)prolyl chloride (TPC). The asterisks indicate chiral centers (asymmetric carbons) in the molecules.

reaction mixtures were incubated for 20 minutes at 65°C, allowed to cool to ambient temperature, and evaporated to dryness, followed by the addition of 50 μ l of ethyl acetate for reconstitution. The liquid contents were then transferred to autosampler vials, and 1 μ l was injected onto the gas chromatograph-mass spectrometer (GC/MS) for characterization, confirmation, and quantitation of the derivatized products of amphetamines. Since PFPA is a non-chiral compound, the stereospecificity of the propionylated derivatives could not be established.

Derivatization by TPC

To establish the stereospecificity, the amphetamines were extracted from re-accessioned samples and derivatized by the TPC chiral probe. Amines were extracted by the procedure described earlier and were derivatized by using 50 μ l of the TPC solution. Following the 20-minute incubation at 65°C, the evaporation to dryness, and the reconstitution in 50 μ l of ethyl acetate, 1 μ l of the contents was injected onto the GC/MS system. TPC has previously been used as a chiral probe in the stereospecific analysis of amphetamines (Fitzgerald et al., 1988; Romberg et al., 1995). The chemical reaction for the derivatization is depicted in Figure 2.

Instrumentation

A Hewlett Packard gas chromatograph (Model 5890) - mass spectrometer (MS Engine; Model 5989) system was used during the analysis. A crosslinked 5% phenyl methyl silicone column (15 m x 0.25-mm i.d.; 0.25-mm film thickness) was used. Helium was the carrier gas with a flow of 1 ml/minute. The injection volume was 1 μ l in the splitless mode, with a purge time of 0.5 minute. The injector temperature was maintained at 200°C. The transfer line was set at 275°C. The acquisition was in electron impact mode of ionization using selective ion monitoring.

For the PFPA derivatives of these amines, the oven temperature was kept at 90°C for 1 minute. It was then increased to 150°C at 8°C/minute and then to 280°C at 40°C/minute. The final temperature of 280°C was maintained for 2 minutes.

For the TPC derivatives of these amines, the oven temperature was set at 90°C for 1 minute, increasing to 240°C at a rate of 10°C/minute. The temperature was then increased to 285°C at a rate of 40°C/minute and kept for 2 minutes.

Calibration Curves

Separate calibration curves for amphetamine and methamphetamine in blood, as well as in urine, were constructed. The concentration range of each of these amines for obtaining the curves was 10.24-1000 ng/ml for blood, while it was 31.25-1000 ng/ml for urine. The regression analysis disclosed that the calibration curves were linear in the given concentration ranges; the correlation coefficient for these curves was 1.0 for both amines in both biological matrixes.

RESULTS

Screening

Routine toxicological evaluation of the submitted samples disclosed the presence of amphetamine class drugs at 867 ng/ml and amphetamine/methamphetamine at 261 ng/ml in the urine. The former value was obtained from the Abbott's ADx® Amphetamine Class assay, while the latter value was from the Abbott's ADx® Amphetamine/Methamphetamine II assay. The screening of the blood by the Roche's abuscreen® assay disclosed the presence of 46 ng/ml methamphetamine. In addition, acetaminophen was found at a level of 4800 ng/ml in the urine by the ADx® assay (Abbott Laboratories, Abbott Park, IL). These values are also given in Table I. No volatiles, combustion gases, or other common drugs were detected in the urine and blood.

Confirmation and Quantitation

Since immunoassays provide semiquantitative values and do not distinguish structurally similar compounds, the biological samples were further analyzed for the characterization and quantitation of these amines by the GC/MS method. The PFPA derivatization method disclosed 320 ng/ml amphetamine and 685 ng/ml methamphetamine in the urine and 52

ng/ml amphetamine and 76 ng/ml methamphetamine in the blood (Table II). Under our GC/MS conditions, both amphetamine and methamphetamine clearly separated from each other. The retention times were 4.82 min for amphetamine and 6.23 min for methamphetamine. The respective deuterated internal standards eluted approximately 0.08 min prior to the corresponding non-deuterated amines. All these amines were characterized by the monitoring of selective ions. Although the internal standards were in racemic mixture forms, no separation of the (-) isomers from the (+) isomers was noted; only one peak was noticed with either internal standard. There were no interfering peaks.

Stereochemical Differentiation

Analyses of the TPC derivatized products clearly suggested that the amphetamines found in the blood and urine were *levorotatory*. Under the instrumental conditions, the (-) isomers distinctly separated from their respective (+) isomers. With the submitted samples, primary peaks corresponding to the (-) isomers of amphetamine and of methamphetamine were

evident. Upon the TPC derivatization, the racemic mixture of deuterated analogs of either amine eluted approximately 0.3 minute apart. Both amines were confirmed and characterized by their retention times and characteristic ions by utilizing the GC/MS system. An example of the peaks of the standards is illustrated in Figure 3. The retention times, along with molecular ions, of the different diastereomers of these amines and of the deuterated internal standards (racemic mixtures) are presented in Table III. As is evident from Figure 4, there was a baseline chromatographic separation of the diastereomers of the internal standards, as well as of amphetamines and methampphetamines in the processed urine sample. However, a relatively small peak was noted at 13.60 minutes after (+)-methamphetamine- d_8 ; the area of the small peak was 3.9% of the (-)-methamphetamine peak. A similar peak of the same proportion was also noted with the blood sample. Such a peak was not detected with the deuterated and non-deuterated TPC-derivatized standards. The observed peak could be attributed to an undetermined substance present in the biological matrixes.

Table I. Screening findings

Analyte	Specimen	Concentration (ng/ml)
Amphetamine Class Drugs	Urine	867
Amphetamine/Methamphetamine	Urine	261
Acetaminophen	Urine	4800
Methamphetamine	Blood	46

Table II. Confirmation and quantitation of amphetamine and methamphetamine, following derivatization with PFPA, by the GC/MS method

Analyte	Specimen	Concentration (ng/ml)
Amphetamine	Urine	320
Methamphetamine	Urine	685
Amphetamine	Blood	52
Methamphetamine	Blood	76

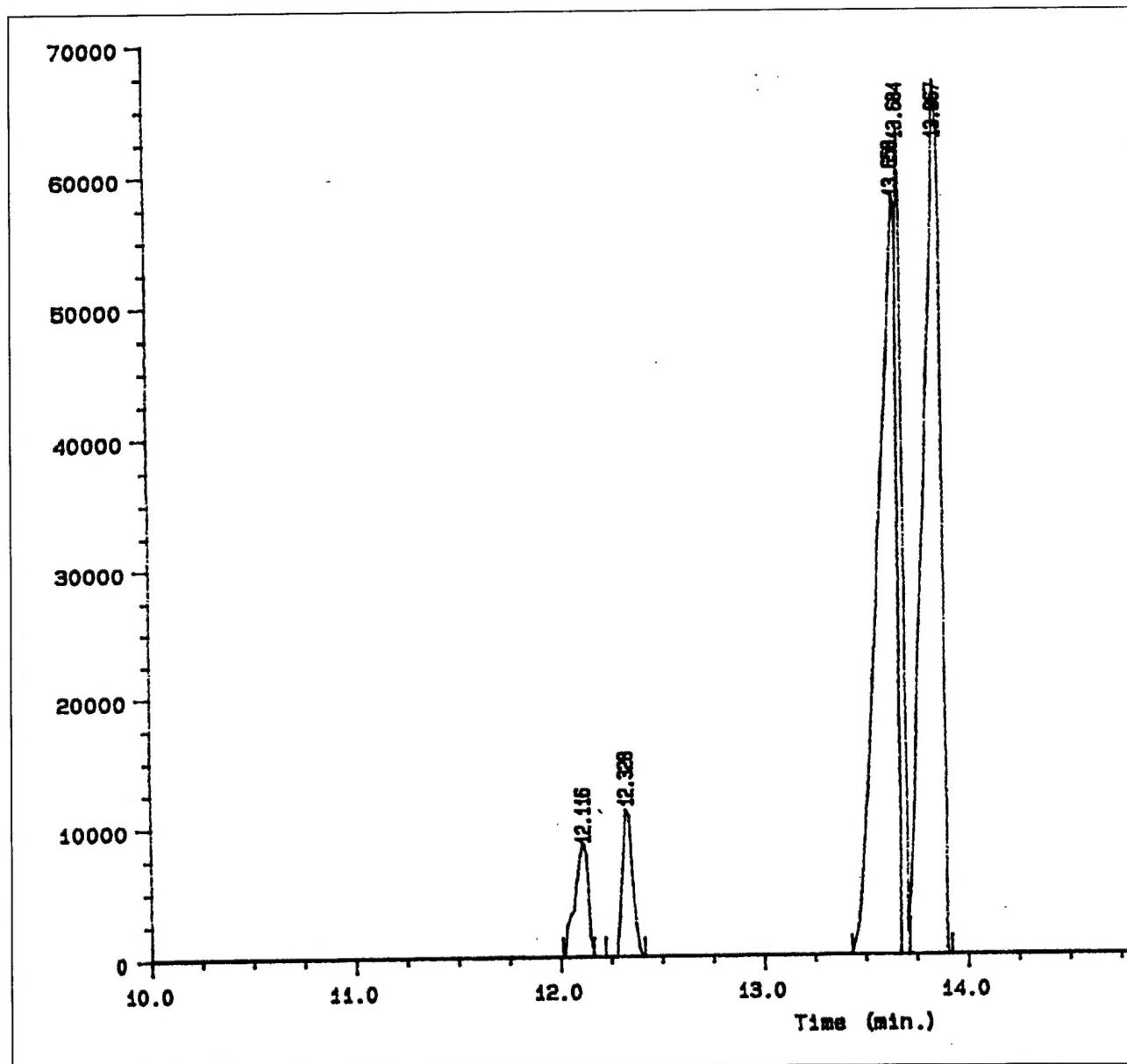


Figure 3. A chromatogram showing separation of standards, derivatized by TPC, of (-) and (+) isomers of amphetamine, as well as of methamphetamine. In the chromatogram, the analytes' retention times (minutes) were: (-)-amphetamine (12.12), (+)-amphetamine (12.33), (-)-methamphetamine (13.65), and (+)-methamphetamine (13.86).

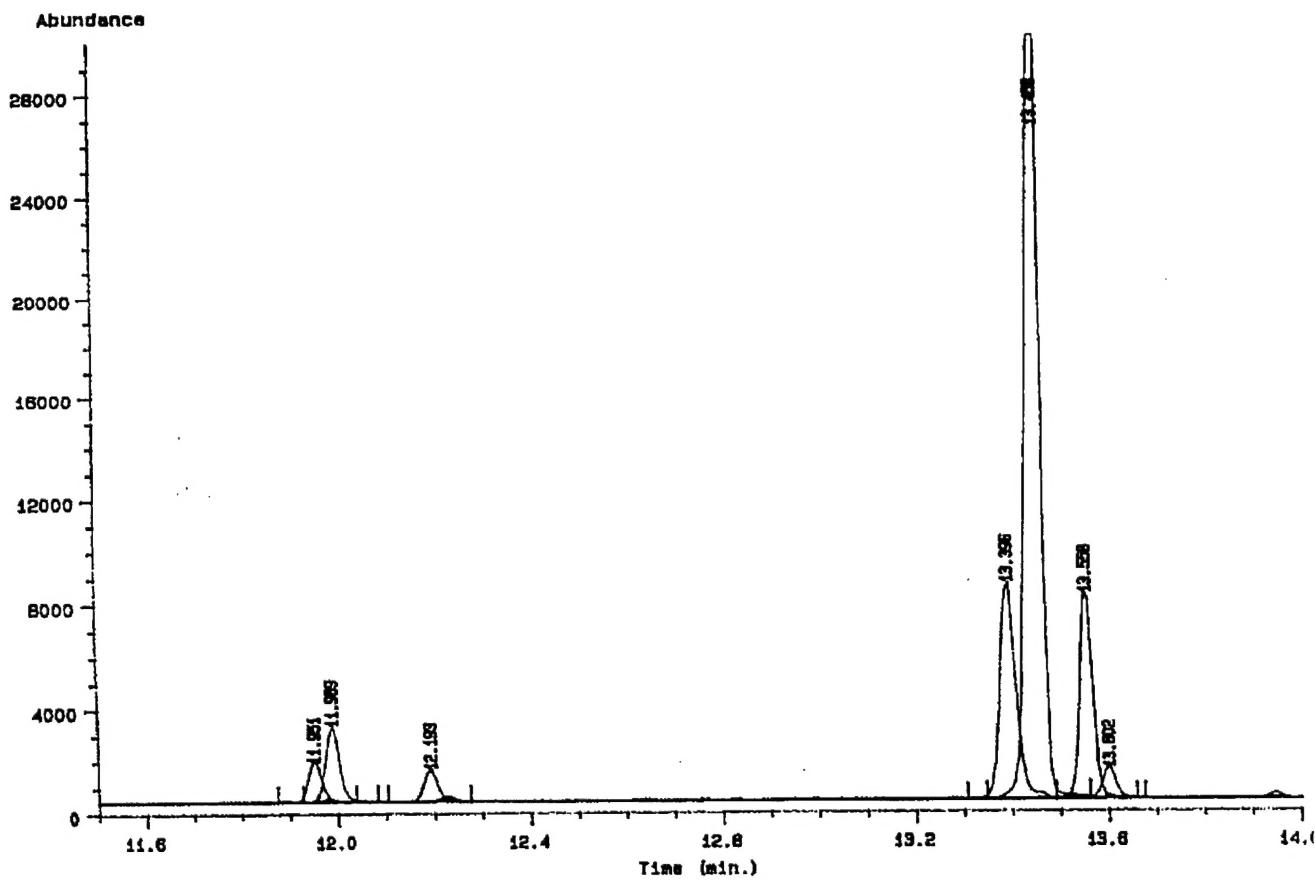


Figure 4. A chromatographic separation of TPC-derivatized diastereomers of (-)-amphetamine and (-)-methamphetamine. Amphetamines were isolated from the urine sample. Deuterated racemic mixtures of amphetamine and of methamphetamine were used as internal standards. Details are described in the materials and methods section. In the chromatogram, the analytes' retention times (minutes) were: (-)-amphetamine- d_8 (11.95), (-)-amphetamine (11.99), (+)-amphetamine- d_8 (12.20), (-)-methamphetamine- d_8 (13.40), (-)-methamphetamine (13.51), and (+)-methamphetamine- d_8 (13.55).

Table III. GC/MS analytical characteristics used in the differentiation of diastereomers of (-) and (+) amphetamine and of (-) and (+) methamphetamine standards utilizing the stereospecific derivatizing agent (S)-(-)-N-(trifluoroacetyl)prolyl chloride (TPC)

Stereospecific amine	Retention time (minutes)	Molecular ion (m/z)
(-)-Amphetamine- <i>d</i> ₈	11.99	240
(-)-Amphetamine	12.05	237
(+)-Amphetamine- <i>d</i> ₈	12.24	240
(+)-Amphetamine	12.31	237
(-)-Methamphetamine- <i>d</i> ₈	13.35	258
(-)-Methamphetamine	13.47	251
(+)-Methamphetamine- <i>d</i> ₈	13.64	258
(+)-Methamphetamine	13.71	251

DISCUSSION

It is imperative to detect, confirm, and quantitate drugs in biological samples to facilitate aircraft accident investigations. Such findings indicate whether the victim was taking any prescribed/nonprescribed medications or illicit drugs; and whether the victim was in compliance with the physician's orders by taking or not taking the medication(s). In those medications where stereospecificity plays an important role in exhibiting pharmacological effects, there is also a need to analytically evaluate whether the compound is *levorotatory* or *dextrorotatory*. Such evaluation is important because only a particular isomer may produce a selective biological response, or a particular isomer may fall under a specific category of the controlled substances. Therefore, the stereospecificity of the analyte should be clearly established for medicolegal purposes.

The toxicological evaluation of the submitted biological samples revealed the presence of *levorotatory* isomers of amphetamine and methamphetamine. Their source could have been selegiline, as this drug biotransforms into these amines. This possibility is supported by the identification of the tablets found at the scene as levodopa and selegiline. Furthermore, the pilot had a medical history of Parkinson's disease and

was being treated for this disorder. Since selegiline is completely metabolized into the three major metabolites, the parent compound is not present in appreciable amounts in the biological system. The demethylated metabolite, (-)-*N*-desmethylselegiline, could not be characterized in the samples, because of the unavailability of its reference standard. However, the chiral derivatizing agent was instrumental in demonstrating the presence of *levorotatory* isomers of these amines in the submitted biosamples. This analytical procedure was able to differentiate *levorotatory* isomers from their *dextrorotatory* isomers. The absence of *dextrorotatory* amphetamine and methamphetamine clearly indicated that the victim did not consume the controlled form of these drugs. If the differentiation between the stereoisomers of amphetamine and methamphetamine had not been made, the individual might have been falsely accused of using the controlled substances.

The concentration ratios of methamphetamine to amphetamine in urine have been used to establish the origin of these amines (Kidura et al., 1992; Romberg et al., 1995). According to Romberg et al. (1995), a ratio of about 2.80 for (-)-methamphetamine to (-)-amphetamine concentration in the urine of selegiline users has been observed. This ratio is close to the 2.14 ratio calculated from the present study, further suggesting the origin of these amines to be selegiline.

(-)-Methamphetamine and (-)-amphetamine could also be present in the urine of the users of Vicks Inhaler™ (Cody and Schwarzhoff, 1993). However, the ratio in such cases usually exceeds 8 (Romberg et al., 1995), thereby ruling out the inhaler as the source of these amines in this case.

In the present study, the concentrations of amphetamine and methamphetamine were derived from the PFPA derivatization method, which does not differentiate the *levorotatory* isomers from the respective *dextrorotatory* isomers. In this method, both (-) and (+) isomers elute at the same time. Therefore, the quantitative values represented the total amounts of both optical isomers. To establish the correctness of the quantitative results, known concentrations of (-)-amphetamine and (-)-methamphetamine were quantitatively analyzed, using the PFPA method and calibration curve. The experimental values for these amines were very close to the expected concentrations. This observation suggests that the PFPA method is suitable for the quantitation of the amines and is independent of their optical property. Therefore, the TPC derivatization method should be used to distinguish the *levorotatory* isomers from the respective *dextrorotatory* isomers. This suggestion is further supported by the fact that only one peak was observed when the deuterated internal standard (a racemic mixture) of either amine was derivatized with PFPA, whereas the racemic mixture derivatized with TPC produced two distinct peaks. These peaks were characterized by the retention times of the respective (-) and (+) isomers of amphetamine or methamphetamine.

SUMMARY

This study suggests that the source of amphetamine and methamphetamine in the samples as being selegiline. This conclusion is based on the stereospecific analysis of these amines in conjunction with the clinical history of the pilot and the presence of two tablets identified as levodopa and selegiline. With optically active medications, the stereospecificity determination is essential for the correct interpretation of the toxicological findings to facilitate accident investigations.

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